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biosyn

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VACMUNE®

LIQUID/POWDER

IMMUCOTHEL®

- Hemocyanin and immunocyanin products as therapeutic agent
- Carrier protein in human vaccines
- Antibody production and immune stimulator

biosyn®

Contents

biosyn: The Hemocyanin People

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Information on use, user responsibility, procurement and shipment of biosyn hemocyanin and immunocyanin product

biosyn manufactures and markets various different grades of hemocyanin and immunocyanin products. These include, research grade, high purity grade and Clinical-GMP grade material.

biosyn clinical grade products, IMMUCOTHEL® and VACMUNE® liquid, are investigational products and as such are provided to customers for whom biosyn has issued a drug master file (DMF) cross reference letter. It is the user's responsibility to ensure that biosyn KLH is suitable for their intended use. biosyn has experience in using the biosyn KLH-product in the treatment of bladder cancer and as such all information provided to customers will be based on our limited experience to date in such patients.

biosyn does not guarantee or assume responsibility for the usage of this product by its customers for other clinical purposes.

Customers may obtain pricing and order the product at the following location:
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Carlsbad, CA 92008, USA.
Phone: (7 60) 4 31-05 90;
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biosyn will ship all products FOB Carlsbad or FOB Fellbach, Germany. biosyn's responsibility of the product shipped stops after the package has been delivered to the carrier of customers choice. We will assume all responsibility until that stage and the responsibility of the product is solely that of the customers. biosyn will arrange for product insurance on behalf of the customer at the time of shipment and package transfer to carrier, if the customer specifically requests or advices biosyn for such insurance. The cost of insurance will be charged to the customer. biosyn liquid products are shipped cold and there is a basic packaging cost associated with all shipments and is determined by the product quantity and value. This cost will be included in the invoice.

General Introduction to Hemocyanins

Hemocyanins are proteins that use copper binding sites to bind and transport oxygen in a variety of arthropods and mollusks. Hemocyanins, like hemoglobin, are multi-subunit molecules where each subunit binds oxygen. Typically, hemocyanins have a high molecular weight; and pH, temperature and ionic concentration modulate the oxygen affinity. The subunit of hemocyanin has a tendency to aggregate.

Hemocyanin is isolated from the hemolymph of the animals. The copper in the protein is in the form of Cu(I) and is bound directly to the amino acid side chain as opposed to the metal being bound to a prosthetic group as in hemoglobin. The oxygenated molecule generates the characteristic light absorbance in the near ultraviolet region around 340 nm, responsible for the blue color of hemocyanins.

The hemocyanins from mollusks and arthropods are quite different in weight, subunit structure and oxygen binding capacity.

Because of its high molecular weight, structural heterogeneity and its completely xenogeneic nature – mollusks and arthropods have been separated from vertebrates as protostomia for at least 600 million years – hemocyanin is one of the strongest antigens known. In mammalians it leads to the formation of very powerful antiserum, moves the T4/T8 ratio in favor of the T4 helper cells and at the site of application leads to local erythema and invasion by macrophages.

Hemocyanin has been in use as an immunological reagent for many years. It is used as a carrier protein for antibody production against antigens. As such, many chemical companies have been marketing the crude and partially refined grade of hemocyanins, specifically the hemocyanin from a mollusk, the Giant Keyhole Limpet, Megathura crenulata (commonly abbreviated as KLH), for over 30 years.

Recent advances in immunology and the role immune system plays in diseases have opened a whole new era of product development activities aimed at developing novel therapeutics which is aimed at teaching the body's immune system to fight diseases like cancer, AIDS, etc. The approach involves the use of highly immunogenic molecule like the hemocyanin for non-specific immunostimulation (NSI) or active specific immunostimulation (ASI) using conjugate vaccines, wherein the tumor (disease) specific antigens are covalently bound to carrier protein like KLH and the product used in human clinical studies. Such products are termed “vaccines”.

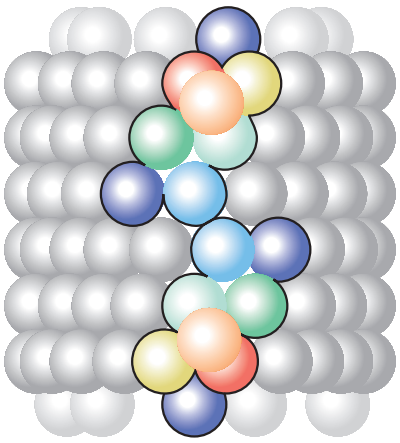
The need for such vaccine development is associated with the production and manufacture of large quantities of safe human products, and the hemocyanin in such vaccines is an active component and should meet the stringent regulatory requirements, i.e. it must be a safe, characterized product of high quality with batch to batch consistency.

The biosyn hemocyanin business plan was initiated in 1985, which included the regulatory and quality aspect built into the development program. As a result of the well-charted development course, biosyn is now the world leader in the manufacture and supply of high quality clinical and research grade hemocyanin products. biosyn hemocyanin products include a variety of different and novel formulations derived from the mollusks giant keyhole limpet and abalone and the arthropod horseshoe crab.

Keyhole Limpet Hemocyanin (KLH)

Keyhole Limpet Hemocyanin (KLH)

The hemocyanin produced from the hemolymph sera of the marine mollusk Giant Keyhole Limpet, Megathura crenulata, has been in use and marketed as a crude or partially purified product for over 30 years by many chemical companies. This molecule is well recognized and commonly abbreviated as KLH. The crude research grade KLH is used in antibody production in animals against antigens. The animal sera containing the antibody for the antigen are further processed and used as immunological reagents or in immunological assays. The partially purified product has also been used in early human trials for immune status evaluation.



KLH Structure

The native KLH is a cylindrical copper containing blue protein with a molecular mass ranging from 8-32 MDa (Million Dalton). Electron microscopy studies reveal presence of two oligomeric forms:

- Didecamers with a molecular mass of 8 MDa, are present in the form of cylinders. They are approximately 35 nm in diameter and 40 nm in length.
- Multidecamers with a molecular mass of 12-32 MDa, present as rod of different lengths consisting of stacked didecamers and decamers. Single decamers exhibit rectangular side views.

The didecamer consists of 20 subunits and occurs in two different forms named KLH1 and KLH2. The subunits are biochemically distinct. Analysis of KLH by native-PAGE gives two characteristic bands, one corresponding to KLH1 with an apparent molecular mass of about 390 kDa (kilo Dalton) and the other corresponding to KLH2 with an apparent molecular mass of 350 kDa. There are eight functional units in the KLH subunits, KLH1 and KLH2. The molecular mass of the functional units is about 50 kDa. Based on this information it is expected that the molecular masses of the KLH1 and KLH2 subunits are approximately 400 kDa. The functional unit (FU) within the subunit contains a binuclear copper binding site that binds molecular oxygen. The copper is in the cuprous [Cu(I)] state, and oxygen binding generates light absorbance in the near ultraviolet around 340 nm and imparts the characteristic blue color to the molecule. The deoxygenated molecule is colorless.

The peptide sequence surrounding the two copper-binding sites is highly conserved, with three copper-liganding histidines in both cases. The active site geometry and molecular architecture of the mollusk hemocyanin differ from those of arthropod hemocyanin. Removal of second copper from mollusk hemocyanin by cyanide ions is both slower and more difficult compared to arthropod hemocyanin, where all the copper come off readily. Further differences between the two hemocyanin subunits are revealed by hydrogen peroxide treatments.

biosyn Native KLH

biosyn Native KLH

The high molecular weight, native KLH, has been extensively over the last several years, and is still being used. Native KLH has been manufactured and supplied by many chemical suppliers.

However, most such KLH are not of the required quality and consistency suitable for use in human clinical trials. Typically, the endotoxin content in such products is very high as the hemolymph sera production is done by cutting open the animals or extraction from dead animals shipped frozen. The product characteristics are also not very good and could lead to precipitation during reconstitution of lyophilized KLH or short shelf-life of one year or less for liquid preparation. It is well documented that either freezing or lyophilization of native KLH leads to loss in activity.

Characterization of native KLH is also an issue as the large molecular weight of 8-32 MDa does not allow use of standard biochemical methods for routine quality control, which is further complicated by the use of excessive metal ions like calcium and magnesium, presumably to stabilize the molecule.

Right from the start of the hemocyanin development work in 1985, biosyn had identified these to be critical issues and developed appropriate strategies to overcome these limitations. To achieve the desired safety, quality and consistency of native KLH, biosyn has developed the non-lethal hemolymph sera collection procedure from live animals and instituted necessary animal handling and quarantine techniques. Quality control methodologies for routine production of this critical raw material have been developed. In addition to this, biosyn has established aquaculture developmental activities, initiated in 1995, to spawn, culture and grow the animals. The biosyn hemocyanin production process is thus environmentally friendly. Moreover, the non-lethal technology allows for the development and institution of appropriate mariculture/aquaculture techniques to ensure future stable source of the raw material. biosyn is currently developing such techniques.

The end result of these technology developments in the animal handling, bleeding and manufacturing process was the production of biosyn low endotoxin and high concentration native KLH of consistent high quality in a phosphate buffer containing no extraneous metal ions for product stabilization, however with improved stability.

The biosyn native KLH is supplied as High Purity Grade and Research Grade. The GMP grade material is ideally suited for use as a carrier protein in the manufacture of vaccines for human use. The Research Grade material is suited for use in vaccine product development activities and also for routine immunological studies, antibody production, production of activated KLH and other developmental activities. The native KLH formulations currently available from biosyn are listed in the table.

Product	Grade*	Uses
Native KLH in phosphate buffer, 25-30 mg/mL, low endotoxin, tested for biological safety, viral clearance and metal ions	HPG	For further processing in KLH-conjugate vaccine manufacture for human and animal use
Native KLH in phosphate buffer, 25-30 mg/mL	RG	Routine immunological and antibody studies and antibody production. Initial product development activities for conjugate vaccines. Preparation of Research Grade activated KLH
Native KLH ammonium sulfate salt, low endotoxin, 20-30 mg/mL	HPG	For further processing and preparation of low endotoxin KLH products
Native KLH ammonium sulfate salt, crude	RG	Low-cost product for antibody production and research studies

** HPG = High Purity Grade; RG = Research Grade*

Immunocyanin: biosyn Proprietary Formulation

The subunits of hemocyanin are defined as Immunocyanin. The Keyhole Limpet Immunocyanin (KLI) are well defined molecules with a molecular mass of about 400 kDa.

IMMUCOTHEL®

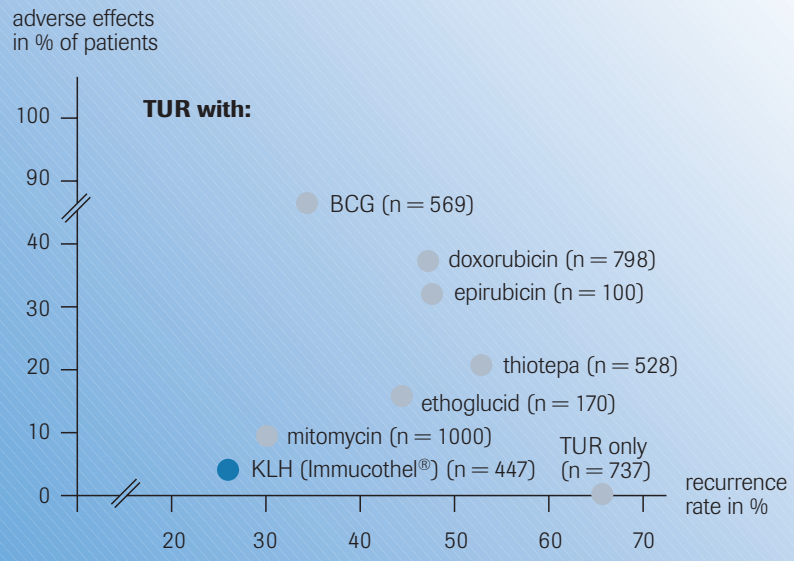
As described above native KLH and hemocyanins in general are aggregates of subunit structure. In view of the variable aggregation or subunit association pattern, the native KLH is a mixture of molecules with varying masses, and are difficult to characterize. These limitations prompted biosyn to investigate the preparation and properties of Immunocyanin. Early studies indicated that there were many advantages to the Immunocyanin compared to the native KLH, from a pharmaceutical product perspective. It was observed that under controlled preparation conditions the Immunocyanin showed biological activity similar to native KLH, however the physical properties were different. The Immunocyanin could be readily prepared in a lyophilized form without loss of activity unlike the native KLH. Such lyophilized product could be readily reconstituted leading to clear preparation. These advantages prompted biosyn to develop its proprietary Immunocyanin products for use in its bladder cancer clinical trials (IMMUCOTHEL®) and also development of novel Immunocyanin formulation as well-characterized carrier protein (VACMUNE®) for the manufacture of vaccines and for general immunological research as well as for research antibody production.

IMMUCOTHEL®: biosyn Immunocyanin in Treatment of Bladder Cancer

Since 1985, biosyn has established a well-defined program for the development of therapeutic products in the area of bladder cancer in particular and cancer in general. IMMUCOTHEL®, containing the active ingredient, biosyn Keyhole Limpet Immunocyanin is the most advanced product investigated for the prevention of bladder carcinoma recurrences, after surgical removal of bladder carcinoma and in cases where other established therapies have failed.

The use of hemocyanin, KLH, in treatment of bladder cancer dates back to 1974. Olson et al. (J Urol 1974: 111, 173-176) reported a markedly reduced incidence of tumor recurrence in 10 patients with superficial bladder cancer who received 5 mg of subcutaneous KLH as a sensitizing dose followed by 200 µg intradermally, to assess cellular immune competence. Since then, the utility of the immunotherapeutic approach in the treatment of bladder cancer has been well established with bacillus Calmette-Guérin (BCG), which is currently the standard of care for carcinoma in situ (CIS) of the urinary bladder and TCC.

IMMUCOTHEL®: Efficacy and adverse effects



IMMUCOTHEL®
Active ingredient: Immunocyanin. **Indications:** Prevention of bladder carcinoma recurrence after transurethral resection and after failure of established therapies for this indication. **Composition:** IMMUCOTHEL® 1 mg: 1 vial with 54.63 mg powder for injections contains 1 mg biotechnically obtained, chromatographically uniform, molecularly standardized immunocyanin. Each vial is accompanied by 1 ampoule with 1 ml solvent. IMMUCOTHEL® 10 mg: 1 vial with 546.3 mg powder for intravesical instillations contains 10 mg of biotechnically obtained, chromatographically uniform, molecularly standardized immunocyanin. Each vial is accompanied by 1 ampoule with 10 ml solvent. Excipients: Powder: Glycine, sodium hydroxide, sodium chloride, sucrose. Solvent: Water for injections. **Contra-indications:** Immunosuppression. Known hypersensitivity to proteins foreign to the body. **Undesirable effects:** Subfebrile temperatures for up to 3 days. Rarely allergic erythema in patients susceptible to allergic reactions. Rarely increase of γ-glutamyl transferase and of glutamate pyruvate transaminase. Urgency. Feeling of pressure or pain. Allergic reactions of the bladder manifesting as sterile leukocyturia. **Interactions:** Specific interactions were not observed. The immunostimulating effect of IMMUCOTHEL® may be impaired by concurrent immunosuppressive radio- or chemotherapy or by simultaneous administration of corticosteroids. **Packages:** IMMUCOTHEL® 1 mg / 10 mg: 1 vial of 1 mg / 10 mg immunocyanin and 1 ampoule with 1 ml / 10 ml solvent. Subject to prescription.
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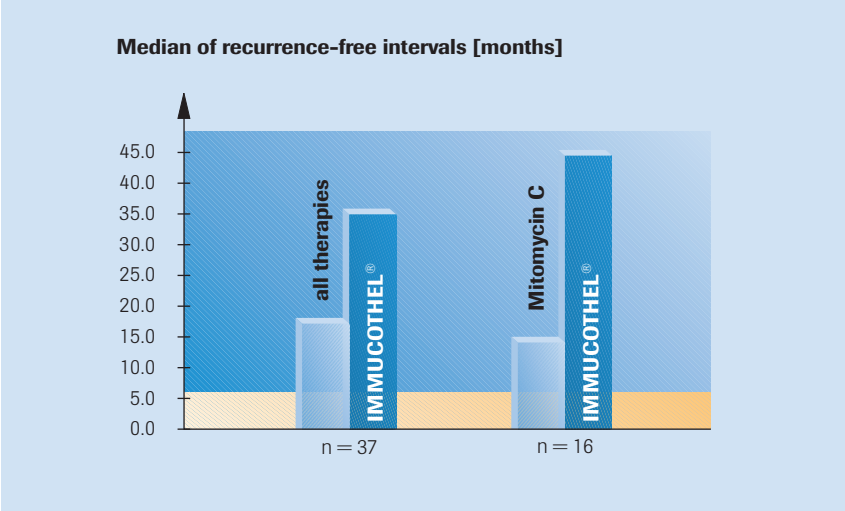
IMMUCOTHEL®

biosyn continued its efforts in the development of its bladder cancer hemocyanin products since its inception in 1985. biosyn's development efforts focussed on product development as well as clinical development of its products. The clinical development efforts were continued with the native Keyhole Limpet Hemocyanin and the product development effort focussed on developing a more homogeneous product of reproducible quality and consistency. These efforts led to the development of Immunocyanin and IMMUCOTHEL®, a lyophilized formulation of Immunocyanin, of desired pharmaceutical quality and activity.

The clinical development of IMMUCOTHEL®, initially performed with the native KLH, has continued to date with the IMMUCOTHEL®, the KLH product. IMMUCOTHEL® was approved for marketing in the Netherlands in 1997 and in South Korea and Austria in 2002. This represented a major breakthrough for the biosyn Immunocyanin product approach as an efficacious product, as well as to the patients, who can now expect to receive a safer treatment.



Intra-individual comparison of the recurrence-free intervals of previous therapies and under IMMUCOTHEL®

IMMUCOTHEL®: Drug Product

IMMUCOTHEL® preparation consists of two dosage forms. The 1 mg dose is used for s.c. immunization and 10 mg dose vials are used for intravesical instillation. The IMMUCOTHEL® 1 mg and 10 mg doses are a lyophilized preparation of Keyhole Limpet Immunocyanin (KLH subunits). The IMMUCOTHEL® is reconstituted with solvent (Sterile Water For Injection, supplied with the kit) and used immediately.

VACMUNE®: biosyn's Immunocyanin as Carrier Protein in Vaccine Manufacture, Antibody Production and other uses

Immune System

Vertebrate animals possess an effective immune system which prevents the invasion of parasites such as bacteria, viruses and cancer cells. The role of the immune system is to specifically recognize and selectively eliminate foreign invaders. Vertebrates are protected by a dual immune system known as cell-mediated immunity and humoral immunity. The cells involved in both immune systems are lymphocytes which originate in the bone marrow and migrate to different lymphoid organs. There are two types of lymphocytes which are known as T cells and B cells. T cells, bone marrow derived lymphocytes that pass through the thymus, are responsible for cell-mediated immunity. B cells, lymphocytes derived from bone marrow but passing through bursa of Fabricius in birds or the bursa equivalent in mammals, are responsible for production of antibodies, which are specific to the antigen, and such immunity is called humoral immunity.

Basic to the humoral immune response is the formation of antibodies, protein molecules, defined as an "immunoglobulin capable of specific combination with the antigen that caused its production in the susceptible animal." Antigens are traditionally defined as any substance that, when introduced parenterally into an animal, will cause the production of antibodies and will react specifically with the antibodies in vitro, which in turn is the basis of a number of immunological assays. Such antigens are macromolecules (MW >10,000 Da) with a high degree of internal chemical complexity. Antibodies are not produced against one's body's own molecules or against low molecular weight molecules (< 10,000 Da).

Haptens are low molecular weight molecules that are non-antigenic and cannot stimulate an immune response by themselves when introduced into the body as a foreign substance. An immune response can be elicited against such haptens when they are combined, chemically or physically, with large protein molecules which basically act as a carrier. Such protein molecules are therefore called, appropriately, carrier molecules.

Immunotherapeutics: New Vaccine Technology

A vaccine involves the administration of an agent to an individual which will stimulate the immune system to react against the "foreign" components of the vaccine. Unlike traditional vaccines, which stimulate the body's immune system to produce antibodies against a potential external infection, immunotherapeutic vaccines involve immunizing a patient so as to cause the immune system to produce antibodies against an internal disease-causing substance, thereby treating the patient's existing condition. In comparison to other therapeutic approaches this potentially has significant benefits, such as specificity of action, avoidance of side effects and long duration of action between booster doses.

Progress in the field of immunotherapy, over the last 15 years, has led to the discovery of a number of potential antigens and antigen delivery systems. Currently a number of these immunotherapeutics are being evaluated in clinical trials to treat diseases like cancer, AIDS, infectious disease, etc.

Of the many different immunotherapeutic vaccine technologies, vaccine formulation using the antigen coupled to carrier proteins has made tremendous progress in the clinic. The carrier proteins that have been used include Tetanus toxoid, Diphtheria toxoid and KLH. The TT and DT carrier proteins were initially used due to their ready availability in the desired quality for clinical use.

Over the last few years, KLH has been routinely used as carrier protein in laboratory animal studies to enhance immune responses to administered antigens. Because of its inherent advantages and, more recently, availability of KLH of clinical grade, it is used extensively in the preparation and evaluation of KLH-antigen conjugate vaccines for a number of disease indications including cancer, AIDS, id-vaccine, infectious disease, vaccines against drug abuse, etc.

KLH is also very extensively used as an adjuvant in nonconjugate vaccine approaches as it is a well known B and T cell activator. KLH has other uses like patient immune status evaluation.

biosyn Immunocyanin Products

The active ingredient, immunocyanin, consists of a stable mixture of two types of protein subunits from native Keyhole Limpet Hemocyanin (KLH). Native KLH is isolated from the hemolymph of the marine mollusk Megathura crenulata using biosyn proprietary technology. The native complex molecule has a molecular weight of 8-32 Million Dalton, and consists of at least 20 subunits. There are two immunologically distinct subunit types, which are composed of eight domains respectively. Each domain contains copper in the heme O_2 binding site. The molecular weight

Product	Grade*	Uses
VACMUNE® liquid, 20 mg/mL A novel formulation of immunocyanin (KLH subunits) in water. The immunocyanin concentration is 20 mg/mL	HPG/CG	• For further processing and in bulk KLH-conjugate vaccine manufacture. Antigens like carbohydrate, peptide, protein, other molecules • Conjugate vaccines for disease types – cancer, AIDS, drug abuse, hypotension, infectious disease and others • As lymphocyte helper antigen and/or immunological tracer molecule, in nonconjugate vaccine approaches, (e.g. for dendritic cell-based bulk vaccine manufacture) • Manufacture of conjugate vaccines for human and animal use
VACMUNE® liquid, 20 mg/mL A novel formulation of immunocyanin (KLH subunits) in water. The immunocyanin concentration is 20 mg/mL	RG	• Routine immunological and antibody studies and antibody production • Initial product development activities of vaccines • Preparation of research grade activated KLH subunits
IMMUCOTHEL® powder, 10 mg and 1 mg A novel lyophilized formulation of immunocyanin, containing 10 mg or 1 mg immunocyanin and sucrose and glycine as excipents	CG	• Clinical studies involving immune status evaluation of patients during various disease stages • Immunological tracer molecule • CD4 helper antigen (nonconjugate vaccines)
VACMUNE® powder, 1 mg and 10 mg	RG	• Low cost product for antibody production and research studies • Pre-clinical studies • For small scale research conjugate vaccine preparation and antibody production • Any application requiring low endotoxin and high purity KLH product

** HPG = High Purity Grade; RG = Research Grade; CG = Clinical Grade*

of the subunits is 400 kDa (kilo Dalton) each.

Physicochemical and biochemical properties of the immunocyanin are as follows:

- Soluble in aqueous buffer at pH 8-10
- Reassociation of subunits occurs at pH 7.5 or below in the presence of calcium and magnesium
- Copper to protein ratio is $1.8–2.5 \times 10^{-3}$
- UV absorption maxima at 279 nm and 347 nm
- Reversible binding of oxygen
- Isoelectric point (pI) ~ pH 5
- Electrophoretic mobility:
  - In native conditions, the two KLH subunits migrate at 350,000 Dalton (KLH2) and 390,000 Dalton (KLH1)
  - Under denaturing conditions, one band is apparent, migrating at approximately 370,000-380,000 Dalton
- Chromatographic size characterization results in a single peak at MW 420 kDa (using Superose™ 6)
- The protein demonstrates an apparent sedimentation constant of 14S

biosyn currently markets a number of user friendly immunocyanin products. The list of immunocyanin products marketed under the trade name VACMUNE® or IMMUCOTHEL® are detailed in the Table.

Other Hemocyanin Products

biosyn has now available hemocyanin and immunocyanin products from abalone and horse shoe crab. These unique products of high quality are useful in research and conjugate Vaccine product development activities.

biosyn Conjugation Service

General Conjugation Procedures – Using biosyn Hemocyanin and Immunocyanin Products

Why Conjugate?

Substances of low molecular weight (haptens) and some high molecular weight proteins alone will not generate antibodies or are not highly immunogenic when injected into an animal. Therefore, the haptens are usually conjugated to a large protein molecule known as a "carrier."

The carrier facilitates the production of antibodies in the following ways:

- The increased size of the hapten-carrier complex is sufficiently large to be recognized and engulfed by the antigen presenting cells of the immune system.
- The protein carrier molecules contain sequences that are T cell helper epitopes that stimulate the proliferation of helper T cells. Interaction of helper T cells with B cells is essential for a strong immune response.
- Since carrier proteins are foreign to the animal, they enhance the response from the immune system.

Choosing a Carrier Protein

The choice of the carrier protein depends on the intended use of the hapten-carrier protein complex. For routine animal studies for research investigation and possibly development of immunological assays, there are a number of proteins that have been used as carriers, including Keyhole Limpet Hemocyanin (KLH), Bovine Serum Albumin (BSA), ovalbumin, and Tetanus toxoid. KLH and BSA are probably the most commonly used.

However, for human clinical studies the most widely used carrier proteins are Keyhole Limpet Hemocyanin, Tetanus toxoid or Diphtheria toxoid. The critical parameters in the selection of the protein are elucidation of the necessary immune response for the intended clinical use and the toxicity of the carrier protein and the conjugate, i.e. minimal secondary complications. These are generally evaluated during the early research and pre-clinical studies. KLH has been the most widely used protein due to the consistent yield of enhanced immune response with a wide variety of haptens and also protein molecules.

KLH is one of the largest foreign proteins that is most different from the human and generates the best immune response. When immunized with a hapten-KLH conjugate, antibodies are also generated to the KLH. Therefore, it is necessary to use a hapten-conjugate that is different from the KLH used for your assays. For example, a KLH conjugate should be used for immunizations and a BSA conjugate for assays. More recent investigations suggest that biosyn hemocyanin from abalone and horse shoe crab may be used as an additional carrier protein.

Why Use biosyn KLH?

biosyn KLH has many advantages over other commercial grade material that can be procured from other vendors. The major distinction lies in:

- Environmentally friendly process used in the extraction of hemocyanin from animals.
- biosyn uses a proprietary methodology and has built-in a test procedure for harvesting and handling of live animals, unlike the use of dead or frozen animals or lethal extraction procedure used by most commercial KLH suppliers.
- biosyn hemocynin and final products are therefore of high purity and low endotoxin quality.
- biosyn KLH subunits, Immunocyanin, are of low molecular weight (400 kDa) and are a completely characterized product.
- biosyn manufactures and packages KLH products to meet specific customer requirements including user-friendly, ready-to-use preparation and packaging.
- biosyn Hemocyanin and Immunocyanin are formulated in either pure buffer systems like phosphate buffer or in water and packaged as small aliquots or bulk quantities. This offers customers major advantages, i.e. flexibility of use, choice of buffer in early developmental studies, and avoidance of issues associated with reconstitution of dry powder product obtained from other vendors.
- biosyn Immunocyanin in water is a unique formulation and overcomes one of the major drawbacks of using KLH in clinical and commercial grade conjugate vaccine products.
- biosyn clinical grade products are extensive tested for safety and lack of viruses including hepatitis A, B, C, vibrio species, rota virus and Norwalk viruses.

Conjugation Procedure: biosyn Hemocyanin and Immunocyanin

Care must be taken when choosing the mode of conjugation to prevent modification of the antigenic sequence. For optimum immune response, it is preferable to conjugate via a terminal residue/functional group. Some of the conjugation methods that can routinely be used for peptides are shown in the following Table:

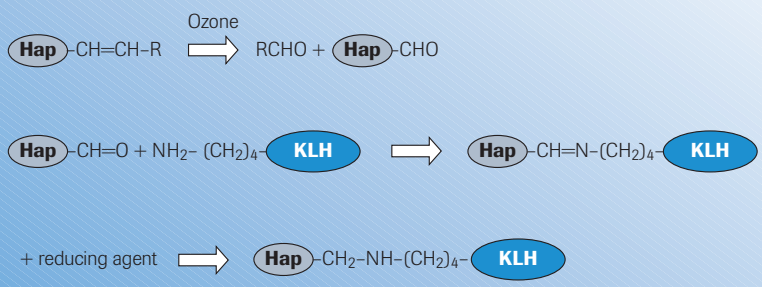
Conjugation methods

Coupling Agent	Reactions	Use Preferentially with Peptides Containing	Avoid if Your Epitopic Sequence Contains*:
Carbodiimides	Couples carboxylic acids to amines to form amides	N-terminal amines C-terminal carboxyls	Aspartic acid, Glutamic acid, Lysine
Glutaraldehyde (Homobifunctional linker)	Adds to amines thiols, indoles, and hydroxyls to form Schiff base polymers	N-terminal amines, N- or C-terminal cysteine thiols	Cysteine, Lysine
MBS, SMCC† (Heterobifunctional linkers) N-terminal amines	Adds to thiols and amines to form thioethers and amides	N- or C-terminal cysteine thiols, Cysteine, Lysine	Cysteine, Lysine

** Except when these residues are located at the terminal portions of the peptide.*
† MBS – m-Maleimidobenzoyl-N-hydroxysuccinimide ester, SMCC-(Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carbonate). These reagents can be procured from Pierce.

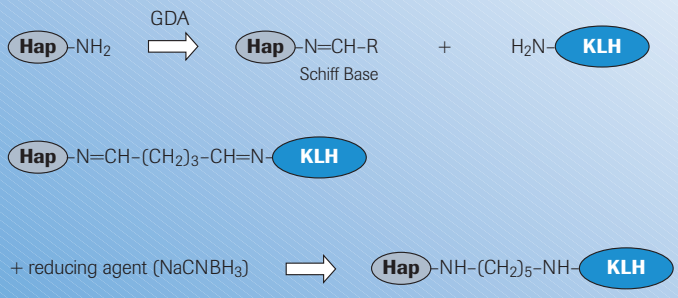
Conjugation strategies for modified KLH VACMUNE® I

Conjugation of carbohydrates – aldehyde functional groups
direct conjugation by reductive amination technique



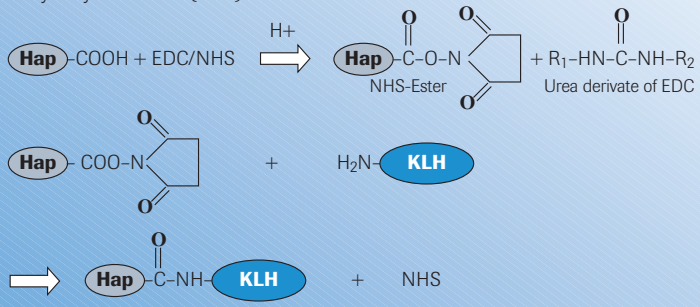
Conjugation strategies for modified KLH VACMUNE® II

Conjugation of N-terminal amines of proteins
conjugation by Glutaraldehyde (bifunctional linker) (GDA)



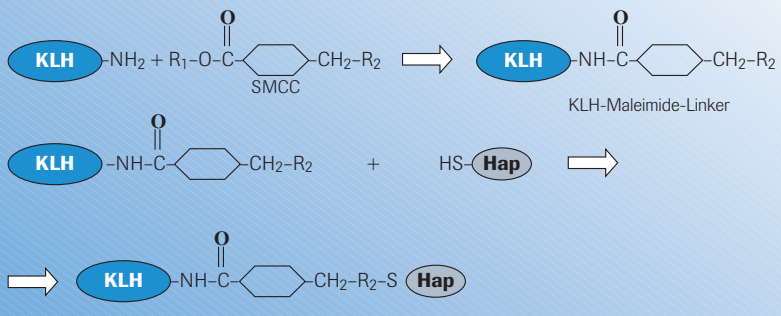
Conjugation strategies for modified KLH VACMUNE® III

Conjugation of N-terminal amines and C-terminal carboxyl groups of proteins
conjugation by 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and
N-Hydroxysuccinimide (NHS)



Conjugation strategies for modified KLH VACMUNE® IV

Conjugation of N- or C-terminal cystein thiols of proteins/N-terminal amines
conjugation by Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC)



Conjugation procedures can be used to couple your peptide to either biosyn Hemocyanin or Immunocyanin. Due to the differences in molecular weight between Hemocyanin and Immunocyanin, separate calculations are needed to determine the amount of peptide or hapten in general to be conjugated. The following Table provides information for such calculation and includes calculation for BSA. This may be required if you need to obtain BSA conjugate for analytical purposes.

General Remarks on Coupling Reactions Using biosyn Hemocyanin and Immunocyanin

Many of the published coupling reaction conditions may be employed for hapten-protein conjugate preparation. Such products in general can be used for routine pre-clinical and immunological studies. However, when preparing Clinical Grade hapten-conjugates, a number of parameters may have to be investigated as these are unique to the given hapten conjugate. There are no universal reaction conditions that would be applicable for all haptens. biosyn hemocyanin and immunocyanin products are unique in that many of the coupling reactions can be carried out simultaneously or in parallel as they provide the necessary flexibility. For example, the KLH subunits preparation (VACMUNE® liquid) is a preparation in water, at high concentration (20 mg/mL) and with no other extraneous impurities. The KLH can be readily put into a variety of buffers, without the need for extensive dialysis as would be the case with other commercial KLH products. Reaction parameters like concentration, buffer composition, pH, temperature etc., which are critical for optimal conjugation, are readily achievable. In essence, product and reaction optimization can be done rapidly.

Moreover, lack of extraneous impurities in the critical component, KLH, is a major advantage from a regulatory perspective as you have to only worry about the contaminants arising during the conjugation procedure of which there is internal knowledge.

Conjugate preparation using heterobifunctional linkers like MBS involves a two-step reaction. Each of these steps needs to be carefully optimized. It may also be noted that the first step in this procedure more or less defines the reaction conditions for the second hapten conjugation.

	KLH (Native)	KLH (subunits)	BSA
Molecular weight	5 x 10^6 (average)	4 x 10^5	67,000
5 mg of protein	1 nmol	12.5 nmol	74.6 nmol

For typical and efficient conjugation ratio, the hapten molar concentration in the reaction mixture should be 40 times the carrier protein molar concentration:

- The formula for a 40-fold excess of peptide for KLH (Native) is:
  (40 x 1 nmol = 40 nmol) x molecular weight of peptide = ng of peptide

  For example, if you use 5 mg of KLH and the MW of your peptide is 2000, then a 40-fold excess = 80 µg of peptide.

- For conjugation with KLH subunits, the 40-fold excess peptide concentration would be:
  (40 x 12.5 nmol = 500 nmol) x molecular weight of peptide = ng of peptide

  The weight of the peptide (MW = 2000) would be 1 mg or 1000 µg.

- The formula for a 40-fold excess of peptide for BSA is:
  (40 x 74.6 nmol = 3.0 µmol) x molecular weight of peptide = µg of peptide

  For example, if you use 5 mg of BSA and the MW of your peptide is 2000, then a 40-fold excess = 6 mg of peptide.

biosyn Conjugation Service

biosyn has expertise in protein conjugation and more specifically in conjugation using Hemocyanin and Immunocyanin products. We provide such services on contract. The advantages of the biosyn conjugation service are:

- Experience
- Well equipped for conjugating and developing analytical methods for novel molecules
- Rapid development service
- Clinical Grade/GMP product manufacture
- Scale-up and commercial manufacturing
- Customer oriented – goal is to meet the defined objective of customer including their timelines
- Cost savings

Please contact biosyn, with your specific request details.

biosyn Regulatory Documentation

biosyn has obtained marketing approval for its lead immunocyanin product IMMUCOTHEL® in the Netherlands, South Korea and Austria. IMMUCOTHEL® has been marketed in the Netherlands since 1997, in South Korea and Austria since 2002.

IMMUCOTHEL® is currently in Phase III clinical trials in Germany for further evaluation of its efficacy in bladder cancer treatment.

biosyn has filed Drug Master Files with the US FDA and Canadian Health Protection Branch, for its lead product as well as for VACMUNE® liquid. Currently, we do not have DMF filed with European authorities, however, would be very happy to provide such services.

Product Request and Cost

biosyn supplies all of its products in bulk as well as smaller aliquots. The product price is based on the required grade and purity. For clinical use and for vaccine preparation biosyn supplies the hemocyanin products under supply agreement. The supply agreement includes our pricing policy, documentation and regulatory support costs and any specific development costs. For details please contact biosyn.

Vaccine Developer‘s and Investigator‘s Responsibility

Customers using biosyn hemocyanin products for clinical (immune status or other clinical applications) use are responsible for ensuring that the biosyn hemocyanin is appropriate for their use. Since the biosyn hemocyanin and immunocyanin are investigational products, it is limited to investigational use.

Marketing Partner

biosyn is looking for marketing partners for co-development and marketing of its hemocyanin product, IMMUCOTHEL®, indicated for treatment of bladder cancer, and a number of second-generation hemocyanin products currently under development. Please contact biosyn for further details.

biosyn is also interested in distribution of its Research Grade hemocyanin products as well as many of its generic and food supplement products. Please contact biosyn Germany or biosyn California for details.

Patent Information

The following patents or applications for patents entitled “Use of hemocyanins and arylphorins to influence the immune system and for the treatment of tumors” have been granted or have been made:

- European patent no. 0320528 (9.8.93),
- U.S. patent no. 5,231,081 (17.07.93),
- Canadian patent no. 1336322 (18.7.95),
- Japanese patent no. 2709111 (17.10.97).

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